PSEUDO-EPIDERMIS: A MODEL SYSTEM FOR INVESTIGATING MOLECULAR AND CELLULAR PATHWAYS OF CUTANEOUS EPIDERMAL TOXICITY FROM SULFUR MUSTARD

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ABSTRACT

Damage to DNA, Inhibition of DNA replication and mitosis, appearance of abnormal keratin peptide and large differentiated cells and, finally, death of cells occur dose- and time-responsively in submerged cultures of keratinocytes exposed to bis-(b-chloroethyl)sulfide (BCES). However, the relevance of these parameters to vesication in human skin exposed to mustard *in vivo* has yet to be established. The pseudo-epidermis cultured from human cutaneous keratinocytes offers a system in which the pathogenic importance of each of these parameters can be evaluated. To establish the validity of the system, it is necessary to show that the pseudo-epidermis undergoes similar dose- and time-dependent cytotoxicity from BCES as is observed in the human skin after topical exposure to the mustard. This report includes data which demonstrate a dose- and time-dependent destruction of the germinative layer in human pseudo-epidermis after topical application of BCES. In addition, data are included to show that DNA is a primary target for BCES in pseudo-epidermis as it is *in vivo*. Also included in this report is a proposed sequence of molecular and cellular events to account for cytotoxicity in the germinative population of the pseudo-epidermis after exposure to BCES.

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INTRODUCTION

Destruction of the epidermal basal layer is a major component of the vesication that results from topical exposure of human skin to bis-(β-chloroethyl)sulfide (BCES). The blister consists of systemic fluid capped by relatively intact differentiated cell layers. Investigation of the molecular and cellular etiology of epidermal cytotoxicity is difficult *in vivo* because of the concomitant inflamma-tion. On the other hand, the pathogenic relevance of data derived from exposure of submerged monolayer or stratified, partially keratinized epidermal cultures is difficult to evaluate. The stratified, cornified culture, generated from isolated primary, germinatively-competent, human keratinocytes on microporous synthetic membranes at the air-medium interface (Bernstam, et al., 1990) has biochemical, morphological and functional features comparable to those of the intact tissue. This pseudo-epidermis (cf., Scavarelli-Karantsavelos, et al., 1990) could be a surrogate for studying the molecular and cellular aspects of epidermal toxicity from BCES if it could be shown that the culture exhibits dose- and time-responsive cytotoxicity similar to the human epidermis.

Data in this report demonstrate that the pseudo-epidermis can be used to evaluate the hypotheses proposed to explain the etiology of epidermal degeneration in skin exposed to BCES. Cells of the basal layer are destroyed without similar effect on the differentiated cell layers when pseudo-epidermis is exposed topically to BCES. The response is dose- and time-dependent and occurs after a delay as is the case in the skin *in vivo*. The response requires a 5- to 10-fold higher level of exposure in the culture than is true *in vivo*. Data are included to show that DNA is an early and highly sensitive target of BCES in the culture as is true *in vivo*. This report also contains a proposed sequence of molecular pathways plus functional and structural changes as a working hypothesis for the progression of events that leads to loss of germinative potential in the pseudo-epidermis - and possibly in the epidermis itself. This postulated sequence can be evaluated in the pseudo-epidermis.

METHODS

Stratified, cornified cultures of keratinocytes (pseudo-epidermis) were grown from primary isolates of germinative-competent basal cells derived from human adult epidermis as described by Bernstam., et al. (1990) with the following modifications: the type of trypsin previously used was replaced with crystalline porcine trypsin (Sigma Type IX; microporous membranes were coated with 0.01% calf skin collagen and bovine pituitary extract and epidermal growth factor were added to the medium. The membrane used as a support for the culture was the Puropor nylon membrane (Catalogue number 66-470, Gelman Sciences, Ann Arbor, MI). The histological techniques utilized are described by Bernstam, et al. (1990). Studies of DNA and protein synthesis by incorporation of radioactive precursors were carried out as described by Vaughan, et al. (1988).

RESULTS

Figure 1 presents photomicrographs of cross sections taken from pseudo-epidermis exposed to 25, 50 and 100 nmol of BCES/cm^2 of culture surface and then further incubated for 24, 48 and 72 hr. After the lowest exposure, no obvious morphological changes are noted until

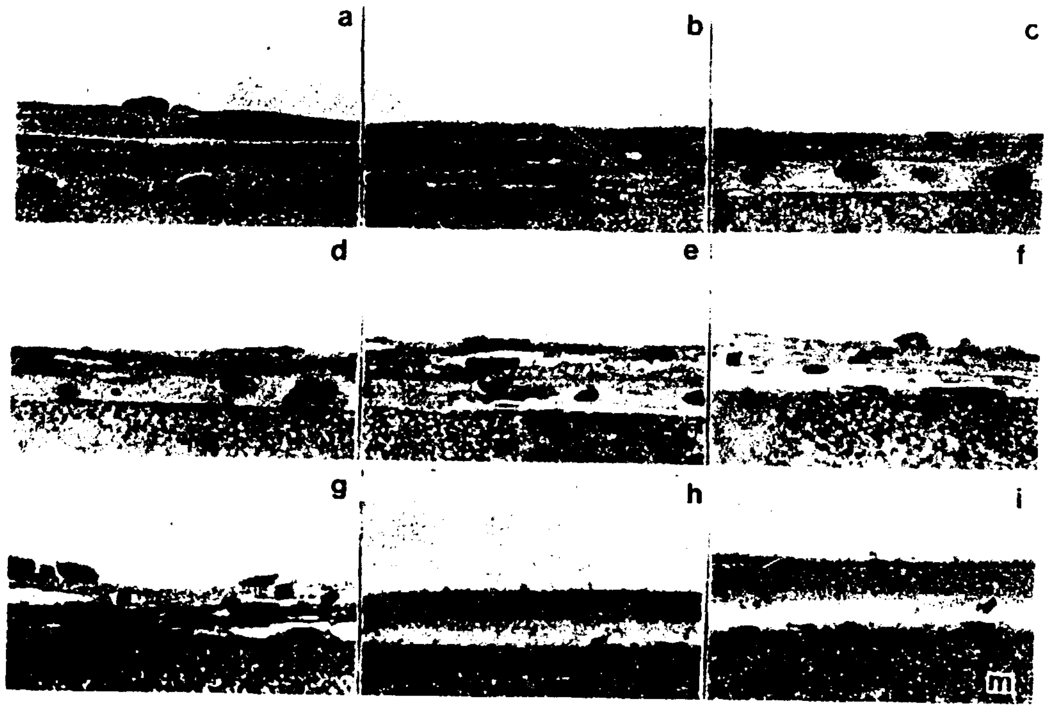


Figure 1. Photomicrographs of human pseudo-epidermis taken at 24, 48 or 72 hr after topical application of 25, 50 or 100 nmol BCES/cm^2 in phosphate-buffered saline. Sections were stained with haematoxylin and eosin. 540x. a, b, c - 25 nmol/cm^2; d,e,f - 50 nmol/cm^2; g, h, i - 100 nmol/cm^2. a, d, g - 24 hr; b, e, h - 48 hr; c, f, i - 72 hr. m - membrane.

72 hr post-exposure (c) when small foci of degeneration were seen in the basal layer. After the intermediate level of exposure, degeneration was more extensive in the basal layer as early as 48 hr post-exposure (e). In the case of the highest exposure, major destruction of the basal layer was seen at 24 hr post-exposure (g).

The data in Table 1 show that the incorporation of [^{3}H]thymidine into DNA was affected at a lower concentration of BCES than was the incorporation of [^{14}C]leucine into protein. The rate of DNA synthesis was 56% of control immediately after exposure to 0.56 nmol of BCES/cm^2 of culture surface. The rate of protein synthesis was 97% of control immediately after an exposure to 1.1 nmol/cm^2. Inhibition of DNA synthesis was still apparent at 24 hr post-exposure but returned to control levels by 48 hr post-exposure. Significant inhibition of protein synthesis was not observed below an exposure of 2.6 nmol/cm with recovery complete at 48 hr post-exposure. Even after an exposure to 1.9 nmol/cm^2, DNA was still only 47% of control at 48 hr post-exposure.

Table 1. Effect of topical exposure to BCES on the incorporation of [^{3}H]thymidine (TdR) into DNA and [^{14}C]leucine (Leu) into protein in human pseudo-epidermis. Radioactive precursors were applied in phosphate buffered saline over the entire culture whose surface was 13.3 cm^2. Determinations were made at 0, 24 and 48 hr post-exposure. N = 5-11.

BCES (umol/cm2)	Control Value (± SD)					
	[^{3}H]TdR			[^{14}]Leu		
	0	24	48	0	24	48
0.19	86±8	94±21	103±26			
0.56	54±10	61±12	96±33			
1.1	23±6	12±6	55±17	97±16	97±4	111±16
1.9				89±10	89±22	109±13
2.6				62±17	68±15	103±14

DISCUSSION

Beginning with the inhibition of DNA synthesis after topical exposure to 0.56 nmol/cm^2 and terminating with complete loss of the basal layer after application of between 50 and 100 nmol/cm^2, the pseudo-epidermis appears to exhibit the complete range of toxicity seen *in vivo* from BCES. Topical application of 0.65 to 6.5 nmol/cm^2 is sufficient to evoke vesication in the human skin *in vivo*. It is not obvious why a larger dose is required to make the pseudo-epidermis respond but Papirmeister, et al. (1984) observed a similar situation when they exposed human skin grafted to the athymic nude mouse. Considering the time- and dose-dependency of the cytotoxicity (cf., Figure 1), it is entirely possible that a dose lower than 25 nmol/cm^2 would have produced more extensive necrosis of the basal layer if the observation had been made at a time post-exposure greater than 48 hr. The data presented in this report encourage the view that the pseudo-epidermis can serve as a model for studying many aspects of toxicity produced in the cutaneous epidermis after exposure to BCES.

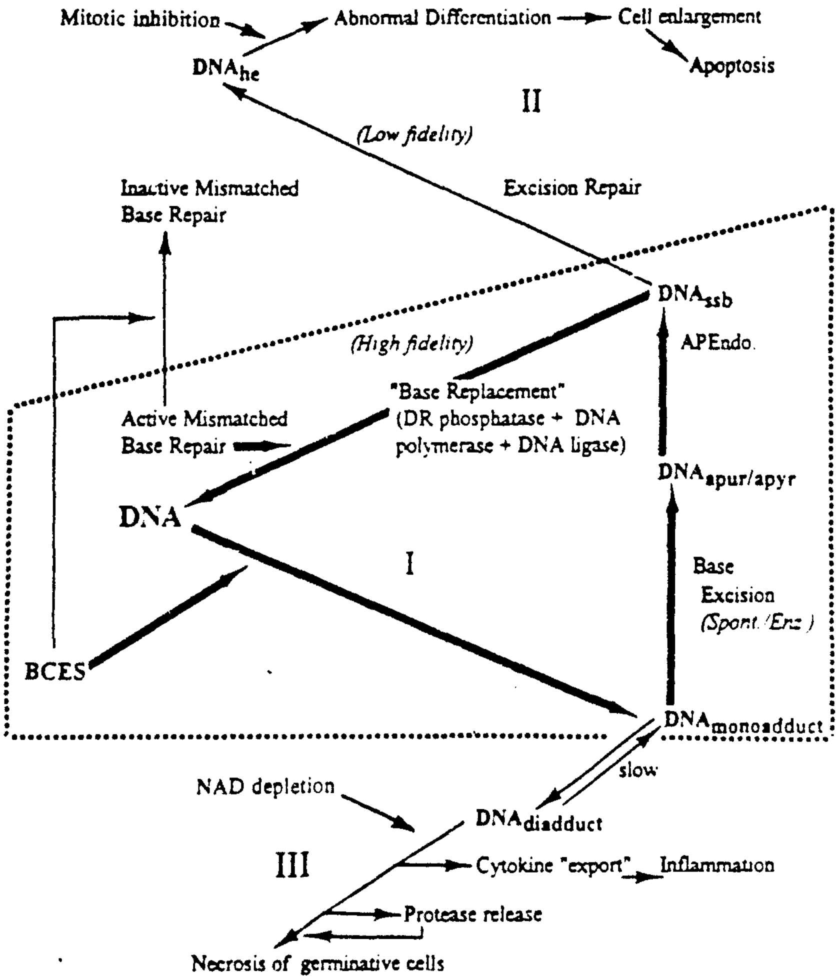
In order to efficiently investigate the molecular and cellular pathways of epidermal toxicity from mustard exposure in the pseudo-epidermis, it would be advantageous to propose a mechanistic scenario which would incorporate available data from submerged cultures of keratinocytes as well as other systems that have been studied. The postulated sequence of events shown in Figure 2 is assumed to take place in the germinative population of the epidermis. The hypothesis includes the "NAD depletion" mechanism proposed by Papirmeister and his colleagues in 1985 (also cf., Papirmeister, et al., 1991) and provides a point of interaction with the inflammatory response (cf., Dannenberg, et al., 1985) as well as the increase in proteolytic activity (cf Higuchi, et al., 1988). Also encompassed is information on repair of DNA, enhanced differentiation and cytotoxicity resulting from the exposure of submerged cultures of rat keratinocytes (cf., Ku and Bernstein, 1988; Ribeiro, et al., 1991; Locey and Bernstein, 1987) and inhibition of DNA synthesis in the pseudo-epidermis derived from rat skin (Vaughan, et al., 1988; Zaman Soroya, et al., 1992).

The hypothesis assumes that toxicity in the basal layer of the exposed pseudo-epidermis goes through three dose -dependent stages in succumbing to complete loss of proliferative potential. It is not possible, at this time, to define with any degree of certainty the range of dosage associated with each stage but they are < 2 nmol/cm^2 for Stage I and > 50 nmol/cm^2 for Stage III applied topically. These values assume that the observations are made at 48 hr post-exposure. Stage I is also defined as a range of exposures from which the germinative population completely recovers without obvious cytotoxicity.

It is proposed that at low concentrations of BCES (Stage I), alkylation of DNA occurs resulting in the formation of mono-adducts which are removed from the DNA by spontaneous and enzymatic depurination/depyrimidation. The deoxyribose phosphate at the apurinic/apyrimidinic sites are hydrolyzed enzymatically producing single strand breaks. Repair is accomplished by "base replacement" i.e., the insertion of the specific nucleotide required by the particular base on the complementary site of the undamaged strand. This is a "high fidelity" process and generally results in a retention of the original base sequence in the repaired strand. If an erroneous nucleotide were to be inserted, the cell's active mismatched base repair system would rectify the error (cf. Fan and Bernstein, 1991) .

At intermediate levels of exposure (Stage II), the concentration of mono-adducts in DNA increases and the "base replacement" repair mechanism is joined by the classical excision repair system. The latter repair mechanism excises oligonucleotide fragments adjacent to the damaged base. Repair, therefore involves the insertion of all four nucleotides and the chance for erroneous insertions is increased. Furthermore, the mismatched base repair system becomes inhibited increasing the chance that misinformation will be inserted in the DNA. The

Figure 2. Hypothetical pathways for cellular toxicity of BCES in pseudo-epidermis.



DNAhe - heterotypic DNA, i.e., DNA with an abnormal base(s). DNAssb - DNA with a single-strand break(s); DNAapur/apyr - DNA having an apurinic or apyrimidinic site(s); DNAmono-adduct - DNA having an alkylated base(s); DNAdi-adduct - DNA having an interstrand or intrastrand cross-link(s); spont/enz - spontaneous or enzymatically catalyzed depurination(s) or depyrimidination(s); AP endo - apurinic (or apyrimidinic) endonuclease; DR phosphatase - deoxyribose phosphatase; NAD - nicotinamide adenine dinucleotide; I, II, III - stages of toxicity (cf. text for definition).

result is heterotypic DNA - DNA with information different than that normally carried by the germinative cell. Cells that were capable of mitosis before exposure, are no longer so (P. P. Lin, Personal Communication) and become committed to differentiation. Once they enter the sequence of differentiation they are programmed for death (apoptosis) and can no longer play a role in maintaining the balance between epidermal proliferation and differentiation. They make abnormal keratin peptide (cf., Locey and Bernstein, 1987), become abnormally large and eventually die. (cf., Ku and Bernstein, 1988)

At high levels of exposure (Stage III), a high concentration of di-adducts (cross-links between chains and between two susceptible bases on the same chain) is formed. Although the cell may have some ability to hydrolyze the di-adduct, the process is slow and the cell faces irreversible cytoxicity. Early in this stage, cytokines are exported to bring on the inflammatory process, NAD depletion occurs because the cell is no longer able to synthesize more of this cofactor and proteases are activated to break up the damaged cell. The epidermis completely loses proliferative capability and, *in situ*, is transformed into the non-functional cap for a blister.

It is likely that the described events are not rigorously restricted to the stage to which they have been assigned and that Stages II and III may occur simultaneously. Because systemic influences including the inflammatory process are excluded from the system, the pseudo-epidermis should be useful in investigating the validity of this hypothetical description of the events in BCES-mediated epidermal toxicity.

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